(12) UK Patent Application (19) GB (11) 2 200 989 (13) A

(43) Application published 17 Aug 1988

(21) Application No 8801876

(22) Date of filing 28 Jan 1988

(30) Priority data
(31) 3703081 (32) 3 Feb 1987 (33) DE

(71) Applicant
Miles Inc

(Incorporated in USA-Indiana)

1127 Myrtle Street, Elkhart, Indiana 46514, United States of America

(72) Inventors
Holger Heidenreich
Herbert Hugl
Klaus Wehling

(74) Agent and/or Address for Service
Carpmaels & Ransford
43 Bloomsbury Square, London, WC1A 2RA

(51) INT CL⁴
G01N 33/52 C12Q 1/00 1/44

(52) Domestic classification (Edition J):
G1B BV
U1S 1333 G1B

(56) Documents cited
EP A2 0198286
Chem. Abs. vl 104 (16) abst 140912
J. Chromatogr. (1980) Vol 187 (2) p418-20
Chem. Abs. Vol 92 (10)87605

(58) Field of search
G1B
Selected US specifications from IPC sub-classes
G01N C12Q

(54) Method and agent for detection of thiols

(57) A method and agent for the detection of thiol groups is presented in a system or formed in a step preceding the detection reaction. This formation can be effected by chemical reactions, such as, for example, reduction of disulphides, or biochemical reactions, such as, for example, thioester hydrolysis by esterases. Fe^{3+} ions are reduced to Fe^{2+} ions by the thiol groups. The Fe^{2+} is then detected with suitable complex ligands, such as ferroins, cuproins and terroins. The complex ligands may also include hydrazones, imines, phenols and tetrazines.

GB 2 200 989 A

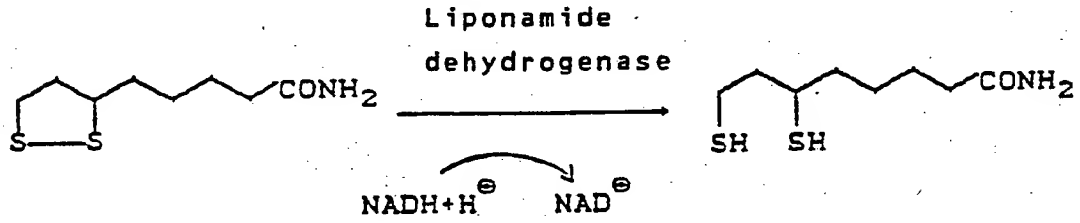
Formulae in the printed specification were reproduced drom drawings submitted after the date f filing, in accordance with Rule 20(14) of the Patents Rules 1982.

2200989

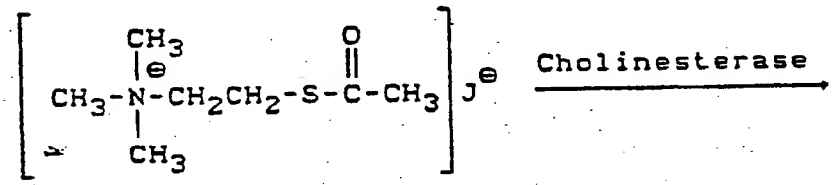
METHOD AND AGENTS FOR DETECTION OF THIOLS

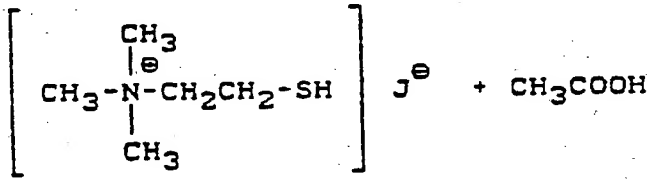
The invention relates to a method and agent for the detection of thiol groups present in a system or formed in a step preceding the detection reaction. This formation can be effected by chemical reactions, such as, for example, reduction of disulphides, or biochemical reactions. The preferred biochemical reactions are illustrated by the following equation:

a)



b)





Numerous methods are known for the detection of compounds containing thiol groups. A particularly useful and frequently quoted method is that described by G.K. Ellman in "Arch. Biochem. Biophys. 1959, 82, 70-77", which is based on the formation of the yellow anion of 3-mercapto-6-nitro-benzoic acid, which is formed by reaction between a thiol and 3,3'-dithio-bis-6-nitro-benzoic acid (Ellman's reagent).

A serious disadvantage of Ellman's reagent is that

only yellow colour shades are obtained by the reduced disulphides. However, colour shades from the red or blue region would be desirable for the visual determination of thiols.

Precisely these colour shades can be developed by the method according to the invention.

The invention is based on the following fundamental reaction:

$$2\ R\text{-}SH + 2\ Fe^{3+} \longrightarrow R\text{-}S\text{-}S\text{-}R + 2\ Fe^{2+}$$

Iron in oxidation level 3 is reduced by thiols to iron in oxidation level 2, the thiols themselves being oxidized to disulphides. The Fe^{2+} ion formed is now converted by suitable ligands into complexes, the molar extinction of which is a multiple of the molar extinction of the Fe^{3+} complexes. In addition, the Fe^{2+} complexes experience a shift in comparison with the Fe^{3+} complexes, so that a clear change in colour can be recognised.

The present invention relates to a test agent for the detection of thiol groups which contains Fe^{3+} ions which can be reduced to Fe^{2+} ions by the thiol groups and a complex ligand which is capable of forming a coloured complex with the Fe^{2+} ions formed.

The detection of Fe^{2+} ions with the aid of suitable complex ligands as coloured complexes is known. Examples of suitable ligands for this are compounds from the group comprising ferroins, cuproins and terroins.

Complex ligands such as hydrazones and their tautomeric azoforms, tetrazolylpyridines, pyridylquinazolines, bis-isoquinolines, imines, phenanthrolines, bipyridines, terpyridines, bidiazines, pyridyldiazines, pyridylbenzimidazoles, diazyltriazines, o-nitroanilines, phenols, tetrazines, triazines, pyridines, phenazine, quinoxalines, benzimidazoles, oximes of substituted methyl or phenyl-2-pyridyl ketones are particularly suitable (Smith, Analyt. Chem., 26 (1954) 1534-1538; Schilt et al., Talanta 15 (1968) 475-478; Schilt et al., Talanta 15 (1968) 1055-1058; and Schilt et

al., Talanta 16 (1969) 448-452.

A description of other complex ligands is to be found in Blandamer et al. J. Chem. Soc. Dalton (1978) 1001-1008, Case, J. Org. Chem., 31 (1966) 2398-2400 and also in English Patent Application 701,843. However, complex ligands other than those mentioned here can also be used.

For use of the test agent and therefore also of the complex ligands in test strips, it may be advantageous for the complex ligands also to carry hydrophobic radicals or ion exchanger functions. Bonding of the complex ligands to the matrix is thereby improved and "bleeding" of the test strip is thus prevented. Hydrophobic radicals which can be used are long-chain alkyl or aralkyl radicals. Polymer bonding of the complex ligands is also conceivable.

The test agent according to the invention can be used for the detection of thiols such as liponic acid amide, thiocholine, glutathione or coenzyme A, or of thioglycosides. The thiol can also be present as a precursor in the form of a thioester, thioether, disulphide or thioacetal in the test agent.

Thus, the test agent according to the invention is also suitable, for example, for detection of enzymes which cleave thio compounds. There may be mentioned here thioether hydrolases, thioesterases or thioglycosidases. However, esterases such as cholinesterase (CHE) can also be detected. The natural substrate of CHE is acetylcholine. However, this enzyme is also capable of cleaving acetyl- and buturylthiocholine. The free thiol group formed by the cleavage is then detected with the test agent.

Biochemical reactions in which free thiol groups are formed on the basis of redox reactions are furthermore known.

There may be mentioned here the reduction of liponic acid catalysed by liponamide dehydrogenase in the presence of reduced nicotinamide ad nine dinucleotide (NADH).

There is therefore the possibility of also using

the test agent according to the invention in the analysis of reactions which depend on NADH. Typical representatives of NADH-dependent enzymes which may be mentioned are: lactate dehydrogenase, alcohol dehydrogenase, glucose dehydrogenase, glycerolaldehyde dehydrogenase, glycerol phosphate dehydrogenase, malate dehydrogenase and the like. The NADH can also be the end product of multi-stage enzymatic reactions, such as in the case of analysis of glutamate oxalacetate transaminase (EC 2.6.11), glutamate pyruvate transaminase (EC 2.6.12) or creatine kinase (EC 2.7.32).

NADH can also be the reaction product in the analysis of substrates such as lactate, glucose, malate, urea and the like. By using the test agent according to the invention in the analysis of NADH-dependent reactions, a substantial improvement in sensitivity is achieved, in particular because two mol of Fe^{2+} are formed per mol of NADH and the coloured complexes in some cases have very high extinction coefficients.

It has proved advantageous to supply the Fe^{3+} complex bonded to the thiol substrate. Possible complexing agents are EDTA, HEDTA, citric acid, malic acid, lactic acid, amino acids, such as, for example, alanine, glycine and glutamine, crown ethers, such as, for example, 18-crown-6, phenylaza-15-crown-5, benzo-15-crown-5 and dibenzopyridino-15-crown-5, or triazinophanes and kryptates, and oxidized sugars, such as, for example, gluconic acid and glucuronic acid.

The concentration ratio of thiol:Fe^{3+} should be at least 1 : 1 and preferably 1 : 5. The ratio of 1 : 20 is particularly preferred for the fastest possible reaction, and the ratio of 1 : 10 is especially favourable.

Test agent or test system in the context of the present invention are to be understood, for example, as those which can be measured in a cell. In addition to the Fe^{3+} ions and the complex ligands, the test agents contain all the reagents necessary for the particular analysis

substance, such as enzymes, substrates, coenzymes, effectors, antigens, antibodies and the like. These test agents can furthermore also contain substances which do not react, such as, for example, buffers, wetting agents and stabilizers. As described above, enzymes which form thiol groups with the aid of NADH and NADPH as the coenzyme are liponamide dehydrogenase and glutathion reductase. Reagent combinations can be prepared from the enzymes, reagents and substances mentioned and are mixed as a solution or as a powder or are in the form of tablets or a lyophylisate. The reagent combination (if it is not already in the form of a solution) is taken up in water or another suitable solvent and a reagent solution is prepared. If the reagent combination consists of individual components, these are to be mixed with one another. After the sample (for example substrate solution, enzyme solution, blood serum, plasma or urine) has been mixed with an aliquot portion of the reagent mixture, the colour formed is measured on a photometer and the particular concentration or substrate concentration is calculated via the molar extinction coefficient and the volumes of reagent or sample added. Both kinetic and end point measurements are possible.

The Fe^{3+} system/ligand can furthermore be impregnated, together with the reagent or reagents or other enzymes necessary for the particular parameter detection, the buffer system, if appropriate wetting agents and activators as well as other auxiliaries, onto absorbent reagent carriers, such as papers, fleeces and the like. For this, one or more impregnating solutions can be prepared in the form of aqueous, organic or mixed solutions, depending on how the reagents or auxiliaries dissolve. Absorbent or swellable carriers, preferably filter paper or absorbent fleece of glass or plastic, are impregnated or sprayed with these solutions. The carriers are then dried. The reagent carriers thus prepared can be used either as rapid diagnostics for direct determination of the contents of liquid (for

example in body fluids, such as blood, urine or saliva, or in foodstuffs, for example fruit juices, milk and the like). The liquid is thereby applied directly to the reagent carrier or this is immersed briefly in the liquid. Semiquantitative determination is possible by allocating a comparison colour to the colour thus formed. Quantitative evaluation can be carried out by reflectance photometry.

It is also possible to introduce the test agent according to the invention into carrier matrices prepared from casting solutions. Examples which may be mentioned here are cellulose, cellulose derivatives, gelatin, gelatin derivatives or plastics, such as polyurethanes and acrylamide. It is advantageous here if the test agent and if appropriate the other necessary reagents are added directly to the casting solution, which means that it is possible to produce the test device, consisting of the carrier and reagents, in one operation.

By eluting the abovementioned reagents with water or buffer or serum from the absorbent carrier, a reagent solution can be prepared, with which substrates or enzymes can be determined in the cell of a photometer as described above.

Suitable buffers for the test agents mentioned are phosphate, citrate, borate and GOOD buffers with alkali metal or ammonium counterions. However, other systems can also be used. pH values which are to be aimed for are 6 to 10, in particular 6.5 to 7.5.

Wetting agents are, in particular, anionic and cationic wetting agents which undergo ionic interactions with the compounds according to the invention. However, nonionic wetting agents which activate the enzymes can also be used. Sodium laurylsulphate, sodium dioctylsulphosuccinate and alkylaryl polyether alcohols are preferred.

Effectors which can be used are those known for the particular enzymatic reaction.

Other auxiliaries which may be appropriate are the

customary thickeners, solubilizing agents, emulsifiers, optical brighteners, constrast media and the like, such as are known in corresponding tests with other chromogens.

Example

Thiol detection with $FeCl_3$ and complex ligands

To detect NADH in the test system described, the following reagent constituents were introduced into a cell:

	Concentration in the test	
1740 µl of 0.1 M/l acetate buffer, pH = 5	87	mmol/l
100 µl of liponamide	2.5	mmol/l
100 µl of $FeCl_3$ solution	1	mmol/l
60 µl of dipyridyl	3	mmol/l
20 µl of liponamide dehydrogenase	12	kV/l

After measurement of the reagent blank values, the reaction was started by addition of 20 µl of NADH solution. The extinction maximum measured is at 515 nm. Kinetic investigations showed a stable end point (change in extinction within 20 minutes = 1%) after a reaction time of only 1 minute.

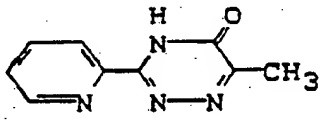
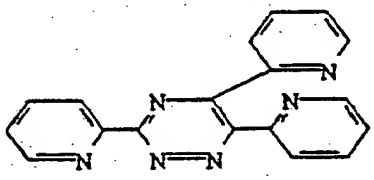
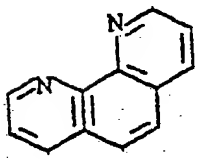
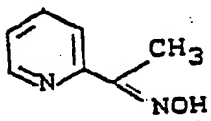
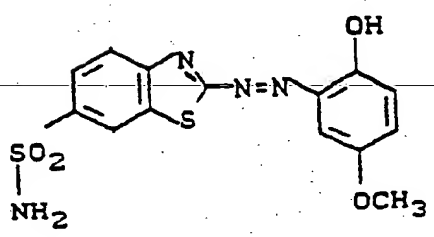
To test the functional capacity and linearity, NADH concentrations in the range from 1 to 10 mmol/l were measured in the test batch. The differences in extinction measured at 515 nm are summarized in Table 1.

Table 1

NADH (mmol/l)	$E_{515\ nm}$
1	0.124
2	0.219
3	0.327
4	0.429
5	0.513
6	0.633
7	0.735
8	0.822
9	0.964
10	1.098

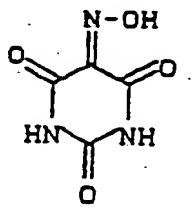
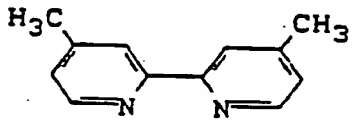
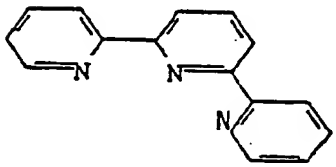
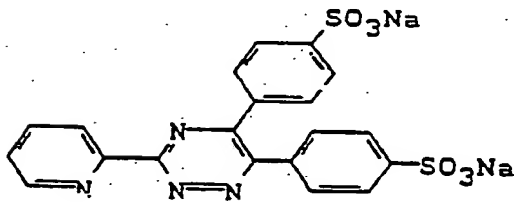
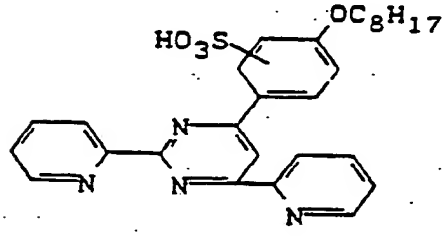
The colours obtainable with the various complex ligands and the corresponding extinction maxima are summarized in Table 2.

Table 2

	Formula	Colour	λ_{max}
1		red	540
2		blue	615
3		red	508
4		red	510
5		violet	556

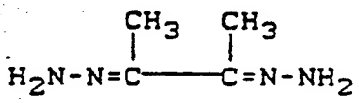
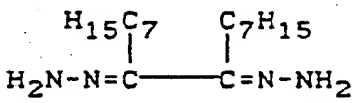
MS 1476

Table 2 (continued)

	Formula	Colour	λ_{max}
6		blue	650
7		red	522
8		violet	552
9		violet	560
10		violet	583

MS 1476

Table 2 (continued)

	Formula	Colour	λ_{max} (nm)
11		yellow	441
12		yellow	443

Patent Claims

1. Test agent for the detection of thiol groups which contains Fe^{3+} ions which can be reduced to Fe^{2+} ions by the thiol groups and a complex ligand which is capable of forming a coloured complex with the Fe^{2+} ions formed.

2. Test agent according to Claim 1 for the detection of thiols from the group comprising liponic acid amide, thiocholine, glutathione and thioglycosides.

3. Test agent according to Claims 1 and 2, containing the thiol as a precursor in the form of a thioester, thioether, disulphide or thioacetal.

4. Test agent according to Claims 1 to 3, wherein the complex ligand for the Fe^{2+} ions originates from the group comprising ferroins, cuproins and terroins.

5. Test agent according to Claims 1 to 4, wherein the complex ligand for the Fe^{2+} ions originates from the group containing hydrazones, tetrazolylpyridines, pyridylquinazolines, bis-isoquinolines, imines, phenanthrolines, bipyridines, terpyridines, bidiazines, pyridyldiazines, pyridylbenzimidazoles, diazyltriazines, o-nitroanilines, phenols, tetrazines, quinoxalines, benzimidazoles, oximes of substituted methyl or phenyl-2-pyridyl ketones.

6. Test agent according to Claims 1 to 5, wherein the complex ligand additionally contains a hydrophobic radical.

7. Use of the test agent according to Claims 1 to 6 for the detection of enzymes which cleave thio compounds.

8. Use according to Claim 7, wherein the enzymes are esterases, thioglycosidases or thioesterases.

9. Use of the test agent according to Claims 1 to 6 for the detection of reduced liponic acid amide.

10. Use of the test agent according to Claims 1 to 6 for the detection of reduced pyridine nucleotides with the aid of liponamides and liponamide dehydrogenase.

11. Method of detecting enzymes, wherein free thiol groups are formed directly or indirectly by the enzymatic reaction and these are detected with the aid of the test

agent according to Claims 1 to 6.

12. Method of detecting substrates, wherein free thiol groups are formed directly or indirectly by the reaction of the substrates and these are detected with the aid of the test agent according to Claims 1 to 6.

13. A test agent according to claim 1, as identified in the Example.

14. A method according to claim 12, substantially as described in the Example.

Published 1988 at The Patent Office, State House, 66 71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office. Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Multiplex techniques ltd. St Mary Cray, Kent Con. 1/87.